

# TISSUE TROPISMS OF AAV VECTORS DEFICIENT IN RECEPTOR BINDING

A Thesis

Presented in Partial Fulfillment of the Requirements for Distinction for the  
Degree Bachelor of Science in the Division of Medical Technology  
in the Allied Medical Professions of The Ohio State University

By

Jennette Kathleen Crumrine

\*\*\*\*\*

The Ohio State University  
2005

Advisors:

Jeffery S. Bartlett, Ph.D., Research Advisor  
Department of Pediatrics and Department of  
Molecular Virology, Immunology, and Medical Genetics  
The Ohio State University

Approved by

Kathy Waller, Ph.D., Honors Advisor  
Associate Professor, Division of Medical Technology  
School of Allied Medical Professions  
The Ohio State University

\_\_\_\_\_  
Advisor  
Division of Medical Technology



## ABSTRACT

Adeno-associated virus (AAV) is a single stranded DNA virus with an icosahedral 20-25 nm capsid comprised of three structural proteins, VP1, VP2, and VP3. AAV is of great interest as a gene therapy vector for the treatment of cancer and genetic diseases due to its ability to transduce both mitotic and postmitotic tissues, be produced at high titers, and mediate long-term gene expression without pathogenicity. However, one concern is that the AAV2 receptor, heparan sulfate proteoglycan (HSPG), is expressed on a wide variety of human tissues. This can cause unwanted gene transfer and expression when only a specific cell type needs to be transduced. Changing arginine amino acids (a.a.) at positions 585 and 588, to alanines (R585A,R588A) has been shown to effectively eliminate AAV binding to HSPG, while the insertion of small peptide epitopes (<20 aa) that bind alternative cellular receptors has been shown to effectively retarget AAV vectors to these new receptors. The amino acid sequence of the major capsid protein VP3 is contained within the two larger and less abundant capsid proteins VP1 and VP2, thus mutations within VP3 are also present in VP1 and VP2. PCR-based site directed mutagenesis of pACG2, which expresses all of the AAV capsid proteins, was used to change the arginines at positions 585 and 588 within the VP1, VP2, and VP3 proteins to alanines. Similarly, these arginine residues were also changed to alanines in a second plasmid that expresses only VP1 and VP3 proteins. Different peptides that target the

vasculature endothelial growth factor receptor 2(VEGFR2) were then incorporated into the R585A, R588A AAV2 capsid constructs. The ability of wild-type AAV2 vectors, R585A, R588A AAV2 vectors, VEGFR2-targeted AAV2 vectors, and an AAV2 vector containing the R585A, R588A mutation on only the VP1 and VP3 proteins to bind to polystyrene beads coated with heparin and to HSPG-expressing Hela C12 cells were assessed. Our preliminary data suggest that AAV2 vectors comprised of capsids with R585A, R588A mutations were unable to bind to HSPG. The wild-type AAV2 was the only virion that successfully bound to the beads and Hela cells. An affinity assay was performed showing that with a competitor in 100-fold excess, AAV2 did not bind as well to the Hela C12 cells similar to the heparan mutants. These preliminary findings suggest that AAV2 does naturally bind to heparan, and that the rAAV2 mutants were deficient in receptor binding. This can lead to successful elimination of unwanted gene transfer and expression and the retargeting of the rAAV2 vector to tissues that were once thought untargetable.

## ACKNOWLEDGMENTS

Dr. Kathy Waller- Thank you for giving me the motivation and self-assurance to do the best that I could. Not only have you broaden my horizons in class, but also with your aspects on life. A positive attitude can always get you far in life. Continue your teaching style and never stop helping students find the right path to follow.

Michele Suhie – Thank you for starting me on my honor's curriculum and assisting in choosing a topic. The guidelines and goals that you gave me really helped overcome that wall of problems/stress. You were always there as a counselor to listen to all of my problems and lead me in the right direction.

Dr. Jeffrey Bartlett- Thank you for having faith in me and allowing me to be a member of your research project. I am very grateful to have had this opportunity to work with you. I will always cherish the experience you have given me.

Matthew Stachler- Thank you for your patience and insightful ideas that guided me in the right direction on numerous occasions in the laboratory. You were always there to help me out of a lot of sticky situations when no one else was. I wish you the best of luck in your future endeavors, for you have the possibility to go far.

Sferra's laboratory- Thank you for your gift of the competitor recombinant virus. I could not have completed my project on time if it wasn't for the small amount of virus needed.

Dr. Wilson - Thank you for your generous gift of paying for all of the Honor students posters.

I would also like to thank my family who made all of this possible through their love, encouragement and sacrifices. You have always taught me to work hard and dream big. I owe all of my great accomplishments in my life to you, thank you.

Finally I want to give thanks to my husband, Bryce, because he was always there for me regardless of the type of day I was having. When things went wrong, you helped me believe in myself and gave me the reassurance that everything would be all right. One thing you have taught me is that everything happens for a reason. Once you open up your mind, body, and soul you can finally listen to your spiritual side.

## TABLE OF CONTENTS

	Page
Abstract.....	ii
Acknowledgments.....	iv
List of Figures.....	vii
Chapters:	
1. Introduction.....	1-4
1.1 Gene therapy and vectors.....	1
1.2 Adeno-associated virus.....	1-4
1.3 Background of problem.....	4
2. Review of Literature.....	5-7
3. Materials and Methods.....	8-15
3.1 Construction of AAV capsid mutant plasmid.....	8-9
3.2 Ligation and Transformation.....	9
3.3 Screening and purifying individual plasmids.....	9-10
3.4 In vitro studies.....	11-12
3.4.1 Cell culture.....	11
3.4.2 Production of recombinant AAV particles with modified capsid....	11
3.4.3 Transfection.....	12
3.5 Purification.....	13
3.6 Virus Titers.....	13
3.7 Infectious Titers.....	14
3.8 Heparin-Binding Assay.....	14-16

3.8.1 Poros Beads.....	14-15
3.8.2 Cell Binding Assay.....	15
3.8.3 Affinity Binding Assay.....	16
4. Research Results.....	17-28
5. Conclusion.....	29-30
6. Bibliography.....	31-32

## LIST OF FIGURES

Figure	Page
1 AAV Capsid.....	2
2 Outer and inner surface map of AAV.....	2
3 Satellitism of AAV with Adeno virus infection.....	3
4 Western Blot of AAV capsid.....	5
5 Visual description of how the three plasmids are placed into the 293 cells and recombine to produce virus.....	12
6 Map of restriction digest for pACG.....	17
7 Photograph of restriction digest separated on 1.1 % agarose gel.....	19
8 Graph of Heparin binding to POROS beads.....	22
9 Graph of Heparan Cell Binding experiment 1.....	24
10 Graph of Heparan Cell Binding experiment 2.....	25
11 Graph of Hela Cell Binding with competitor.....	27



## CHAPTER 1

### INTRODUCTION

#### 1.1 Gene therapy

Gene therapy is an experimental treatment being investigated to correct defective genes that are responsible for disease development. Researchers are studying several approaches of gene therapy to treat cancers and genetic diseases. One of the biggest challenges in gene therapy is finding a suitable vector to carry DNA into tissues. Problems for certain vectors in gene therapy include: provocation of immune response, not invading both quiescent and dividing cells, lack of indefinite expression and producible high titers (1). Leading candidates for gene therapy vectors are the adenovirus, retrovirus, and recombinant engineered adeno-associated virus (rAAV). The adeno-associated virus (AAV) may be a solution to the problems with gene therapy (1, 2).

#### 1.2 AAV

The development of gene transfer vectors from the human parvovirus, adeno-associated virus (AAV), has provided an efficient and effective way of delivering genes into mammalian cells. AAV genome is encapsulated as a single stranded DNA molecule of 4.7 KB. The AAV capsid is comprised of three structural proteins VP1, VP2, and VP3 in an approximate ratio of 1:1:18 (3,4,5,6). The non-enveloped virion is icosahedral in

shape and is one of the smallest (20-25 nM diameter) that has been described. Figure 1 demonstrates the size and shape of the AAV vector courtesy of <http://www.scripps.edu/mb/goodsell/>.

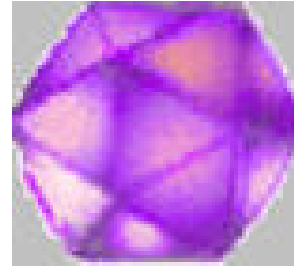


Figure 1: AAV capsid

Even though humans are a natural host for the wild-type AAV virus and at least 80% of the human population is seropositive, no known diseases have emerged from its infection (7). An electron cryo-microscopy and image reconstruction of adeno-associated virus type 2 is shown below in figure 2. Picture A is a view of the outer surface of the capsid and B is the inner surface. The surface maps are colored in such a way to demonstrate the depth of the surface, higher radii are shown in red and lower radii appear blue (8).

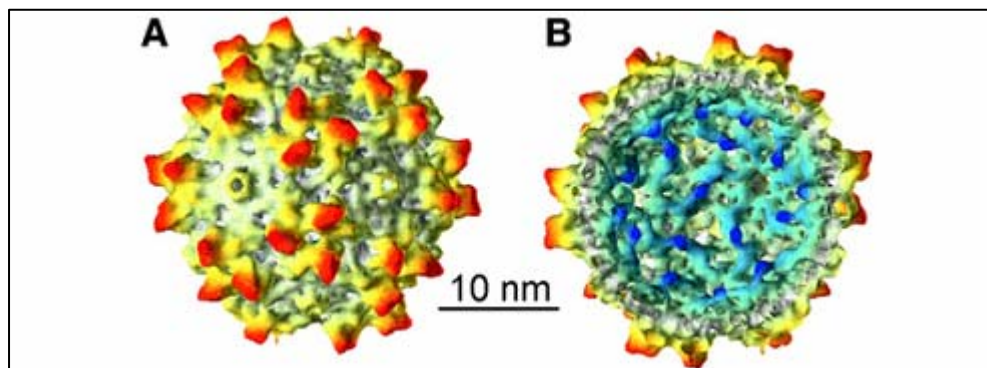


Figure 2: Outer and inner surface map of AAV(8).

Advantages of AAV as a gene delivery vector include its ability to transduce both mitotic and postmitotic tissues, be produced at high titers, and mediate long-term gene expression without pathogenicity (2). AAV is a non-pathogenic defective member of the

human parvovirus family that requires co-infection of a helper virus to propagate itself into the host genome. Figure 3

demonstrates the human adenovirus with adeno-associated virus (arrow), a satellite virus.

AAV requires co-infection with a helper virus to cause a productive infection (9). In the absence of a helper virus, AAV will integrate itself into the host, establishing latency throughout the full life cycle of a cell (10).

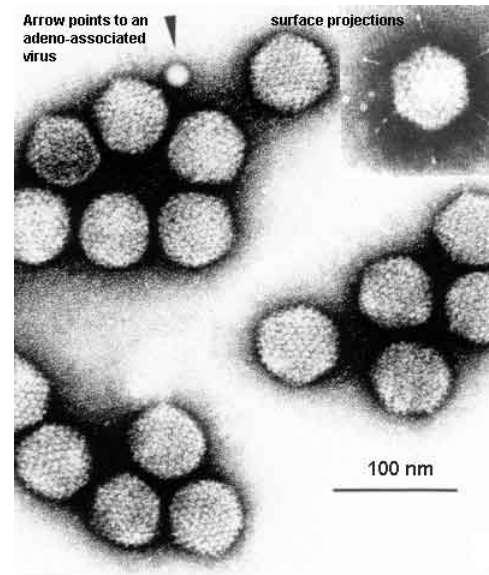


Figure 3: Satellitism of AAV (arrow) with Adeno virus infection (9).

AAV is stable in its latent form and can be rescued to enter into a lytic phase upon infection with a helper virus. In its latent form, AAV based vector is capable of long-term, high-level gene expression even in competent hosts without showing any cellular immune response or toxicity (11). This is practical because only a single injection of a therapeutic gene is required; therefore, no re-administering is necessary. Furthermore, re-administering the virus will increase the chances of the host to produce antibodies against the gene vector, rendering it ineffective.

Another aspect of AAV is its ability to transduce both mitotic and postmitotic tissues such as the lungs, intestines, neurons, muscle, and hematopoietic cells. Even though AAV vectors seem to be a nearly ideal means of genetic therapy, major limitations for clinical application still exist. Limitations of the AAV vector include its inability to target all tissue types, redirecting viral vectors without unwanted transfer of genes and expression, and incorporation of peptides into the viral capsid. The size of

ligands that can be inserted into the AAV capsid is limited due to its small capsid size of 20-24nm (1,2).

### 1.3 Background of problem

In gene therapy, adeno-associated virus is used to deliver a transgene into the body. One of the major concerns with AAV2 as a gene therapy vector is the fact that its receptor, heparan sulfate proteoglycan (HSPG), is expressed on a wide variety of human tissues (1, 2, 3). This can cause unwanted gene transfer and expression when only a specific cell type needs to be transduced. Further clinical development of AAV based gene therapy will require developing AAV vectors with the ability to target specific tissue types. This can be accomplished by the elimination of binding to HSPG and the incorporation of targeting peptides directly into the AAV capsid. By eliminating the virus from utilizing heparan receptors on its capsid protein through mutations, specific sites can be targeted without unwanted gene expression. Point mutations and an addition of small peptides can control how much AAV accumulates in organs of non-interest and successfully retarget the virus to specific sites (3).

## CHAPTER 2

### REVIEW OF LITERATURE

The AAV capsid is composed of three structural proteins VP1, VP2, and VP3 in an approximate ratio of 1:1:18 (3, 4, 5, 6). Figure 4 is a western blot of the AAV capsids, VP1, VP2 and VP3 showing the concentrations of each capsid. The capsids were detected by using monoclonal antibodies that recognizes all three capsid proteins. Certain regions of the AAV vector capsid protein can tolerate the insertion of exogenous peptides. One such example has been the

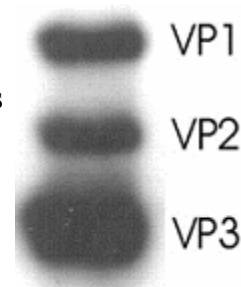


Figure 4: Western Blot of AAV capsid (8).

insertion of an Arg-Gly-Asp (RGD) peptide following amino acid 588 in the normal AAV capsid protein sequence. This RGD peptide allows the modified AAV vector to invade via an interaction with cell surface integrin receptors and without the use of HSPG. By adding the RGD to the virus, one does not need to worry if HSPG is present (2). RGD insertion into the wild type capsid only enhances the chances of the virus to bind to cells. RGD can still bind to heparan, thus in order to redirect the virus, an elimination of HSPG receptors on the capsid is necessary.

Efforts to improve the packaging capacity of the recombinant AAV vectors are still being investigated. Currently, only about 4.5Kb of DNA can be efficiently packaged

into an AAV particle. Understanding the primary steps of viral entry will also be important in defining parameters for efficient gene delivery.

The tropism of AAV can be changed by genetically introducing a ligand peptide into the viral capsid, redirecting the binding of AAV to other cellular receptors. By introducing a mutant insertion into the capsid protein of AAV, we can retarget the tropisms of the virus to cells that are normally resistant to AAV infection (12,13). AAV has been mutated at numerous capsid protein subunits, one site being arginine 484 (R484) and another being arginine 585 (R585). These mutations of the AAV capsid have been shown to drastically reduce heparan binding. Tissue distribution in mice of the double mutated AAV at R484 and R585 indicated marked infection reduction of the liver, compared to infection with the wild-type AAV, but showed an increase of infection in the heart (14). Various mutations sites have been undergone to the AAV capsid, but the specificity and sensitivity of the mutated virus to bind heparan sulfate proteoglycan in-vitro and in-vivo has never been demonstrated.

Current research has demonstrated that AAV can utilize different binding sites other than HSPG to deliver transgenes into the body. Changing arginine amino acids (a.a.) at positions 585 and 588, to alanines (R585A, R588A) has been shown to effectively eliminate AAV binding to HSPG (3, 14, 15). The insertion of small peptide epitopes (<20 a.a.) that bind alternative cellular receptors has been shown to effectively retarget AAV vectors to these new receptors. The amino acid sequence of the major capsid protein VP3 is contained within the two larger and less abundant capsid proteins VP1 and VP2, thus mutations within VP3 are also present in VP1 and VP2. Addition of small peptides (<20 a.a.) that bind specific receptors after amino acid 588 should

effectively retarget rAAV. Another approach involves the incorporation of larger proteins (< 30 kDa) into the capsid into the minor capsid protein region VP2. The VP2 capsid protein region is nonessential and can tolerate large peptide insertions at its N terminus. This will minimize the disruption of the major structural protein, VP3 (6). What is apparent is that there is a lack of correlation of heparan binding and infectivity of some heparan-binding mutants. This means that HSPG might facilitate and enhance AAV cellular interaction and infectivity, yet appears not to be necessary as a receptor site (14). Further testing needs to be done to confirm that various point mutations that reduce the binding and infectivity of the AAV capsid does not bind to heparan sulfate proteoglycan. Once the tissue tropisms of AAV vectors deficient in receptor binding have been investigated, the virus can be retargeted to tissues of interest for gene therapy treatment.

## CHAPTER 3

### MATERIALS AND METHODS

The effects of various mutants that change receptor binding of AAV particles of tissues in direct contact with blood and tissue fenestrations large enough for vectors to enter (liver, lung, spleen, and bone marrow) was monitored. Different mutant viruses were made with mutations in the AAV capsid that allowed infection independent of HSPG and with peptide inserts for retargeting to tissues of interest. Dr. Bartlett's lab has developed methodologies for production and purification of AAV vectors discussed below.

#### 3.1 Construction of AAV capsid mutant plasmid

The plasmid pACG2 (16) was used as the template for all mutant constructions. ACG contains the AAV genome, less the two viral inverted terminal repeats (ITRs), and an ATG-to-ACG mutation of the Rep 78/68 start codon. This mutation has been shown to increase recombinant AAV vector yield by attenuating Rep 78/ 68 synthesis (17). Sites for mutagenesis were chosen on the basis of a computer-generated model of AAV structure. Point mutations at the VP3 region on 585/588 where an arginine has been change to an alanine were undertaken. This prevented the binding of AAV2 to heparan and allows an insertion of new peptides to retarget the vector.



Another type of mutation is the insertion of peptides. Two PCR primers were designed that contained the sequence of the desired insertion plus a unique endonuclease restriction site flanked by 15 to 20 homologous base pairs on each side of the insertion. The PCR products were digested with DpnI endonuclease to eliminate the parental plasmid template. DpnI digests methylated DNA, which degrades the parent plasmid. Mutant plasmids that were made in the PCR are unmethylated.

### 3.2 Ligation and Transformation

The plasmid were ligated together by T4 ligase and transformed into bacteria (DH5- $\alpha$ ), to allow screening of individual plasmids. The PCR purified ligated plasmid was transformed by adding it to competent bacteria into an electroporation cuvette. The cuvette is placed into an electroporation unit where two pulses of 1800 volts will be passed through it. Time constants were monitored to assure recombination. The ligated bacteria will be grown in liquid media (SOC) and then plated onto a selective media plate with selection marker (AMP).

### 3.3 Screening and purifying individual plasmids

Colonies from plates were picked and grown in liquid media (LB or 2X-YT) over night. The liquid bacteria will then go through a mini-prep procedure as determined from QIAGEN. The bacteria will be purified and lysed to leave only DNA. Once the plasmid DNA is purified it will be digested with specific restriction enzyme endonucleases and ran out on a gel. This allows for screening of the plasmids to assess if ligation of the peptides of interest were incorporated into the DNA plasmid. This step

can be repeated numerous times until a digestion that looks promising arise or changes in mutations that should have been added demonstrate an extra band. An agarose gel is made to the thickness desired of a one percent concentration. 1x TAE is used in combination of powder agarose to make a gel. The liquid is heated and mixed until the agarose dissolves and is cooled to about body temperature. Ethidium bromide is added to the one percent solution in a ratio of 1 $\mu$ l per every 20 ml of mixture. Combs are added to produce wells for the digested plasmid to enter. Loading dye is added to the plasmid that will allow us to see the bands once ran out. A base ladder is added to the sides to assess where the bands are migrating and whether or not the bands are the correct base pair. Any mini preps whose digestion looks promising are sequenced to confirm the proper mutant plasmid map. The sequences are read carefully to assure that the mutant was added in the correct site with no other unwanted mutations in the capsid.

Sequences that were correct were grown in a larger amount of media (500ml) and a maxi prep procedure from QIAGEN was preformed. The maxi prep procedure is very similar to the mini prep, but only a larger amount of volume is used. The purified plasmid will be placed in a spectrophotometer and read at wavelengths of 260 and 280 to find out the quantity and quality of the plasmid prep. 260  $\lambda$  tells us the concentration and the ratio of 260 to 280 $\lambda$  tells us the purity of the sample. All plasmids are stored in a -20°C freezer until further use. A portion of the liquid media with bacteria/plasmid growth is mixed 50/50 with glycerol and stored in the -86°C freezer until more plasmid is needed. Once all the plasmids that are required for viral preps are prepared and purified, tissue cultures can be started beginning the in vitro studies of the project.

### 3.4 In vitro studies

#### 3.4.1 Cell culture

Low passage number Hela C12 cells, Hela cells, and HEK 293 cells (17) were grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100U/ml), and streptomycin (100U/ml) at 37 °C and 5% CO<sub>2</sub>. Once the desired confluency was obtained, tests can be preformed or the cells can be split using trypsin to aid in the breaking of the adhesion of the cells to the bottom of the plate.

#### 3.4.2 Production of recombinant AAV particles with modified capsids

To produce rAAV with mutant capsid proteins, 293 cells were transfected with three plasmids. The three plasmids used were as follows: (1) pTR-UF5, which contains the enhanced green fluorescent protein (eGFP) gene driven by the cytomegalovirus (CMV) promoter and flanked by the AAV terminal repeats; (2) the pXX6 plasmid, which supplies the adenovirus helper gene products in trans to allow rAAV production in an adenovirus-free environment (16); and (3) the modified pACG plasmid, which supplies the mutant capsid proteins. As a control, rAAV was also prepared with unmodified pACG plasmid to make the wild type. The plasmids were mixed at a 1:3:1 molar ratio. Plasmid DNAs used for transfection were purified with a Quiagen (Valencia, CA) Maxi-prep kit according to the supplier manual.

### 3.4.3 Transfection

Transfections were carried out as follows. 293 NC2 cells were split 48 hours before transfection so that they could reach confluency around 75% at the time of transfection. Four 10-cm plates were transfected at 37°C, using the calcium phosphate transfection protocol standardized in Dr. Bartlett's laboratory, and incubated at 37°C (19). Forty-eight hours after transfection, cells were harvested by centrifugation at 1200rpm for 10 minutes, resuspended in TMN (200mM Tris pH 8, 10mM MgCl<sub>2</sub>, 1.5M NaCl) and freezing and thawing three times released virus. The crude lysate was clarified by centrifugation at 4200rpm for 20minutes and treated with Benzonase (Sigma, St. Louis, MO) at 50-U/ml final concentrations at 37°C for 30 minutes. Virus was further purified by iodixanol step gradient (20). Figure 5 demonstrates the three plasmids that are added to the 293 cells. After 48-72 hours, the virus is harvested out of the cells and then purified.

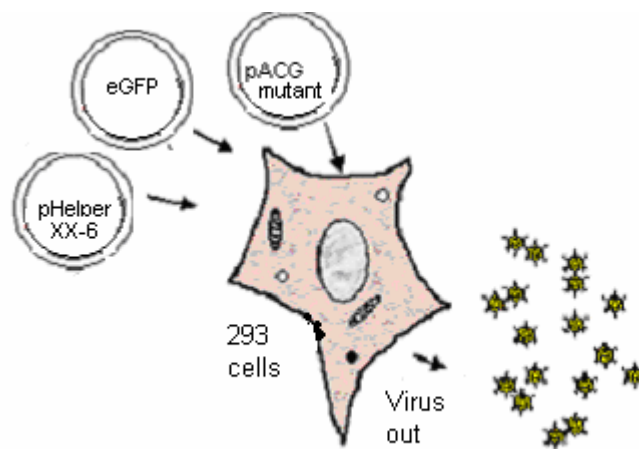


Figure 5. Visual description of how the three plasmids are placed into the 293 cells and recombine to produce virus.

### 3.5 Purification

Once the virus has been harvested from the cells by a freeze thaw method, it can be further purified through an iodixanol gradient. Different concentrations of iodixanol is made and layered from highest concentration to lowest to a centrifuge tube (Quick-Seal Ultra Clear). 60% iodixanol is placed onto the bottom of the tube, followed slowly, trying not to mix, by decreasing concentrations (40%, 25%, and 15%). The clarified lysate/virus is added to the top of the tube and then spun at 350,000 x g for 1 hour at 18°C with a low brake. The tube is carefully removed and is aspirated with an 18-gauge needle at the 40% layer removing only the 40% solution. The virus will migrate to the 40% layer due to weight/ density and proteins and salts will stay in the 25% layer.

### 3.6 Virus titers

The concentration of DNA-containing particles was determined by performing a real-time (RT) PCR using a Perkin-Elmer (PE) – Applied Biosystems (Foster City, Calif.) Prism 7700 sequence detector system. Briefly, 1µl of the purified rAAV stock was diluted in 99µl of digestion buffer (10mM Tris-HCl [pH 8.3], 5 mM MgCl<sub>2</sub>, and 50 mM KCl ) containing Dnase I (350µg/ml). Samples were incubated at 37°C for 30 minutes, then 95°C for 10 minutes. Proteinase K was added to a final concentration of 1 µg/ml and the samples were incubated at 37°C for 30 minutes. Two and a half microliters was used for RT-PCR in a Taqman reaction mixture and completed in compliance as described by Clark (21). The only values that were accepted was within

the linear portion of a standard curve having a coefficient of linearity greater than 0.98. The average RT-PCR titer was calculated from virus preparations assayed two times.

### 3.7 Infectious titers

Infectious titers of rAAV containing wild type or mutant capsids were determined by gene transduction assay. Briefly, HeLa C12 cells were seeded in 24-well plates the day before infection so that they would reach about 75% confluency the next day. Serial dilutions of wild type and mutant preparations were added to the cells in the presence of Ad5 at a multiplicity of infection (MOI) of 3. The cells and viruses were incubated at 37°C for 48 hours. Cells expressing eGFP transgenes were detected by fluorescence-activated cell sorting (FACS) analysis. Infectious titers were also determined in the absence of adenovirus with no differences in the relative titers of mutants.

### 3.8 Heparin-binding assay

#### 3.8.1 POROS Beads

The ability of mutants to bind heparin sulfate was assessed by precipitation with heparin sulfate affinity resin (POROS). Briefly, 293 cells that were transfected and contains wild type and mutant capsids were centrifuged at 4,600 rpm for 3 minutes and the pellet was resuspended in a 500µl of lysis/binding buffer which contains TMN as described before with the addition of 0.5% DOC, Benzonase (50 unit/ml), and 10µl of POROS HE-20 resin. The samples were incubated for 15 minutes at 37°C to ensure complete cell lysis and then gently mixed at 25°C for 2 hours. The resin was washed three times with a wash buffer (12.5 mM Tris, pH 8.0; 0.5mM

MgCl<sub>2</sub>; 100 mM NaCl). The virus was eluted by adding 500µl of elution buffer (20 mM Tris, pH 8.0; 1mM MgCl<sub>2</sub>; 600 mM NaCl) and then a Taqman/DRP analysis was performed as described previously. Controls included precipitation of vectors with wild-type capsids and precipitation of vectors with unconjugated resin.

### 3.8.2 Cell Binding Assay

The ability of mutants to bind to the cell surface of HeLa cells utilizing heparan sulfate was assessed. Testing the ability of mutants to bind to HeLa cells versus heparin coated plastic polystyrene beads will give an efficient and more realistic measurement of binding. HeLa cells have been proven to express heparan on their cellular surface in cell culture. The cellular binding assay is testing the ability of the virus to bind to cells, most likely using HSPG. HeLa cells were harvested and centrifuged at 2,000 x g for 3 minutes and the pellet was washed once with DMEM without serum and then twice with ice-cold binding buffer (DMEM with 2mM glucose, 10 mM HEPES [pH 8] and 1% BSA). AAV vector/virus was added at the highest MOI as possible. The samples were incubated for 60-120 minutes at 4 °C occasionally mixing gently. After incubation, the samples were washed and resuspended in 1x TMN buffer with 0.5% DOC and Benzonase (50 unit/ml). The samples were incubated for 15 minutes at 37°C to ensure complete cell lysis and then spun to clarify the lysate. Taqman/DRP analyses on various serial dilutions were performed.

### 3.8.3 Affinity binding assay

The ability of mutants to bind heparan sulfate on Hela cells was assessed in combination with a competitor. Similarly to the cell-binding assay, viruses were tested to determine if they could bind to the surface of the Hela cells. After cell lysis, the lysate was analyzed by a Taqman/DRP titer to determine the amount of virus in the solution. The viral mutants in our laboratory contain a CMV promoter that drives the enhanced green fluorescent protein and allows the lab to determine the concentration of virus in Taqman. A CMV probe is used in the Taqman/DRP analysis procedure that will pick up any amount of virus in a solution tested. The competitor virus was a gift from Sferra laboratory, which does not contain the CMV promoter. Thus the Taqman CMV probe will not pick up the virus. The virus CC119 from Sferra's lab is a rAAV2 Eflα eGFP at a concentration of  $7.6 \times 10^{12}$  DRP/ml. The affinity assay was performed following the cell-binding assay, only when the viral mutants were added, as was the competitor. The competitor was added in an excess of 100-fold. The procedure was continued exactly as the cell-binding assay.

All methodologies used in this study are well established in Dr. Bartlett's lab at CRI. The appropriate AAV plasmids, antibodies, and other reagents were all on hand or commercially available. Dr. Bartlett is an assistant professor in the Department of Pediatrics and Department of Molecular Virology, Immunology, and Medical Genetics at The Ohio State University.



## CHAPTER 4

### RESEARCH RESULTS

Point mutations and peptide insertions were successfully added to the pACG2 plasmid. PCR-based site directed mutagenesis of pACG2, which expresses all of the AAV capsid proteins, was used to change the arginines at positions 585 and 588 within the VP1, VP2, and VP3 proteins to alanines. The map in figure 6 shows some of the restriction sites. Endonuclease enzymes cut the plasmid in desired places and inserts/ mutations were added.

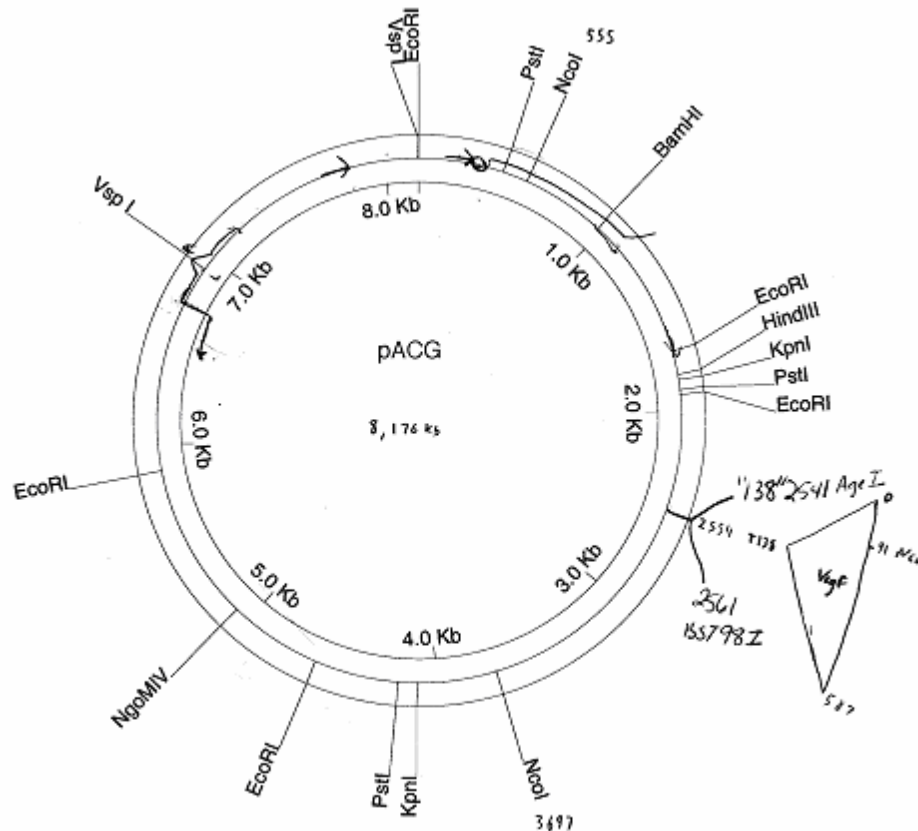


Figure 6. Map of restriction digest for pACG.

The vector plasmid with a 585/588 point mutation of an arginine to an alanine of the VP1, VP2, and VP3 capsid was screened, made, and peptide inserts were successfully added. Peptides inserted that are of interest were a vascular endothelial growth factor receptor 2 (VEGFR2) peptide. This would retarget the AAV virus to the vasculature, which is of interest in our laboratory. Wild-type AAV does not transduce the vasculature very well. Many potential benefits by targeting the vasculature can be envisioned for gene therapy of cancers and cardiovascular diseases. Peptides include: VEGFR2-A (VR2-A), VR2-B, VR2-C, VR2-D, and VR2-E. The peptide sequences for the inserts were added, screened, and made. The inserted peptides will be displayed on all three viral capsid proteins (VP1, VP2, and VP3). This allows for a large number of peptides to be displayed, but only a limited size of peptides can be added. Below is the actual peptides inserted into the 585/588 mutated pACG capsid.

VEGFR 2 A (VR2-A)

```

5' CC GGT TGC GCC ACC TGG TTG CCC CCC CGC TGC GGC
3'      A ACG CGG TGG ACC AAC GGG GGG GCG ACG CCG AAT T

```

VR2-B

```

5' CC GGT TGC TTG CCC CCC AAC CCC ACC AAG TGC GGC
3'      A ACG AAC GGG GGG TTG GGG TGG TTC ACG CCG AAT T

```

VR2-C

```

5' CC GGT TGC TAC GCC ATC ATG CCC TTG GTG TGC GGC
3'      A ACG ATG CGG TAG TAC GGG AAC CAC ACG CCG AAT T

```

VR2-D

```

5' CC GGT TGC ATG CAC TCC GAC ATG CAC GCC CCC GTG TCC GAC ATC TGC GGC
3'      A ACG TAC GTG AGC CTG TAC GTG CGG GGG CAC AGG CTG TAG ACG CCG AAT T

```

VR2-E

```

5' CC GGT TGC CAC ACC ATG TAC TAC CAC CAC TAC CAG CAC CAC TTG TGC GGC
3'      A ACG GTG TGG TAC ATG ATG GTG GTG ATG GTC GTG GTG AAC ACG CCG AAT T

```

The pACG plasmid was cut with enzymes BST 98 I and AGE I for the above VR2 peptides. The peptides VR2A-E were ordered with matching sticky ends shown

above as the underlined peptides, to match the cut plasmid. The peptides were annealed into the capsid of the plasmid with the point mutations of the arginine amino acids (a.a.) at positions 585 and 588, to alanines (R585A, R588A), and then ligated back together in a PCR unit. The ligations were transformed into dsDNA, grown, and mini prep to look for clones. The mini preps were digested with the enzymes BST 98I and VSP I, and ran out on a gel. Figure 7 shows a few of the VR2 samples that were ran out and examined.

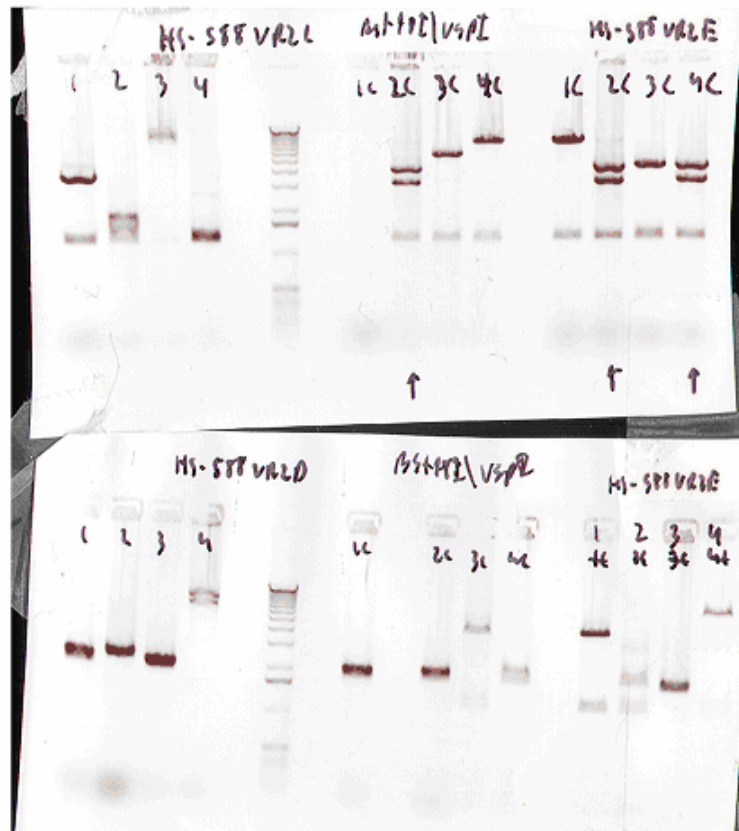


Figure 7: Photograph of restriction digest separated on 1.1% agarose gel. The vectors were cut using BST 98 I and VSPI enzymes in the restriction digest.

The mini preps with arrows are the bands that looked promising based off of the ladder, and were further sequenced to confirm the mutation.

Mutations of the AAV2 capsid were also undertaken in only the VP1, VP3 portion. VP2 was removed so larger peptides could be inserted into the plasmid, but

only displayed a limited amount of times. One of the pitfalls of the AAV capsid is its small size, so it cannot withstand large peptide insertions. What is apparent is that the VP2 capsid is not necessary for viral production. An AAV2 HS- VP1, 3 regions can be combined with a separate VP2 region and still make virus. The VP2 protein can withstand large insertions of peptides into its N-terminal region, like the VEGF receptor. A larger VEGF insertion into the VP2 region has been under investigation, but is still to be made. This would knock out heparin binding and allow the rAAV vector to be retargeted to an area of interest. Once we get the VP2 regions with larger peptides to incorporate properly back into the capsid protein, we can target areas in the body with AAV2 that has never been targeted before.

HS-RGD plasmids were also made where an insertion of an Arg-Gly-Asp (RGD) peptide following mutation of R585A, R588A in the normal AAV capsid protein sequence. This RGD peptide allows the modified AAV vector to interact with cell surface integrin receptors and without the use of HSPG. Current research has demonstrated that the RGD peptide can successfully enhance the binding capacity of rAAV regardless if HSPG was present. Unfortunately the RGD insertion in the laboratory is not working and is currently under investigation. The HS- RGD AAV does not bind heparan but it also does not infect cells either.

All of the mutated plasmids were transfected into 293 cells, harvested, purified and tested for its ability to infect cells. Viruses were DRP titered to determine their concentrations. A 24 well plate with 90% confluent Hela c12s cells were infected with a MOI of 1000 of cell lysate virus of wild-type AAV, HS- AAV, and a control with no virus were tested. Adenovirus was added to half of the wells with an MOI of 10 to show

the increase infectivity when a helper virus is present. The wild-type virus infected the HeLa c12 cells the highest with the helper virus and also without a helper virus. Only a few cells were infected with the HS- AAV with helper virus and none without the helper virus. The control was absent of infectivity with and without the helper virus. The mutated viruses were tested for its ability to bind HSPG.

The ability of wild type AAV2 vectors, and heparan mutants AAV2 vectors to bind to polystyrene beads coated with heparin was assessed. Every assay was performed in triplicates to assure no variations between tubes. A wild type AAV2 vector, a heparan mutant AAV2, and a control were run with every test. Variations in the original protocol had to be undertaken due to unstable results. The original protocol called for viral preps as crude lysates and after numerous repetitions of the assay, variations had to be undertaken. Crude lysates is the viral particles straight out of the harvest cells. The viral preparations are very “crude” in that cell debris, proteins, and salts are also present in the lysate, which was a possibility for the unstable results. All of the crude lysate viral preparations undergone iodixanol gradient purification which gave more reliable results with lower standard deviations between samples.

Viruses that were tested for heparin binding included: wild-type AAV2, R585A R588A AAV2 vectors, VEGFR2-targeted AAV2 vectors, AAV2 vector containing the R585A, R588A mutation on only the VP1 and VP3 proteins, RGD AAV2 vectors, and R585A R588A RGD AAV2 vectors. The RGD vectors did not behave as envisioned, bringing on another twist onto why the vector was not infecting cells. RGD AAV2 vectors used to work in the lab by binding to cells whether heparan was present or not. This would aid the AAV2 vector in binding when heparan sulfate proteoglycan may not

be present in the body. Unfortunately, the RGD vector recently stopped working and investigation has been undertaken to troubleshoot the dilemma. The entire RGD vector runs on the heparin resin were ignored due to the inconsistencies. All of the viral vectors were allowed to bind to the polystyrene beads coated with heparin, washed, and eluted off. While over 63 percent of the wild-type AAV2 was recovered in the elution, less than 1 percent of the heparan mutants were found in the elution. Figure 8 is a graph of the binding for an assay with wild type AAV2, R585A R588A AAV2 VR2B, and AAV2 R585A R588A VP1, 3 plus VP2.

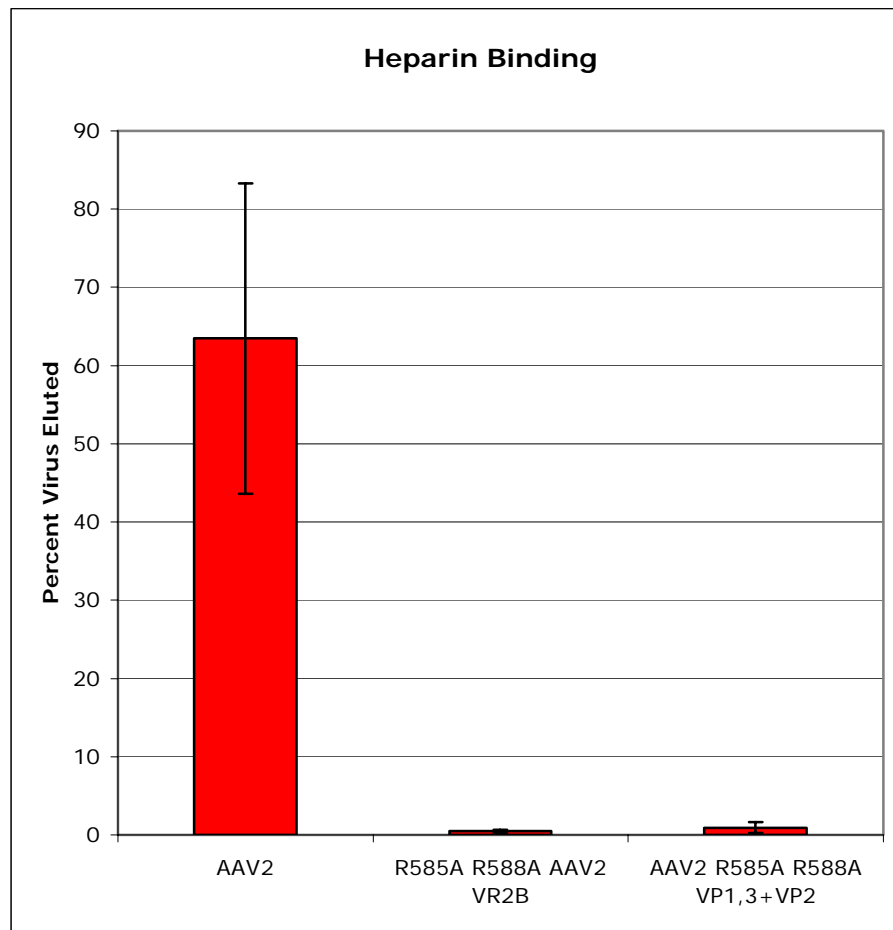


Figure 8: Heparin binding using polystyrene beads coated with heparin and iodixanol purified virus. Error bars indicate the standard deviation between the triplicate samples of each virus. Wild-type AAV2 bound 63% of the time while the R585A R588A AAV2 mutants bound less than 1%.

The above results demonstrated that I successfully knocked out heparan binding in the AAV2 vectors. The viruses were next tested for their ability to bind to heparan sulfate proteoglycan in a more realistic measurement. Cell binding of the viral preps to Hela cells were examined for heparin binding. First experiments were run with  $2.5 \times 10^5$  cells, which produced a weak pellet and were barely visible for washing. After the first run, it was decided to start with  $5.0 \times 10^5$  cells for a larger, visible pellet. All cells were spun at  $2,000 \times g$  for three minutes and then washed with ice-cold DMEM without serum one time. Next the cells were washed two times with a Binding Buffer and then resuspended in a minimal volume. The AAV vectors were added to the appropriate tubes at the determined MOI. The MOI for the first run with only  $2.5 \times 10^5$  cells was 10,000 and MOI for second run was 5,000, resulting in  $2.5 \times 10^9$  total virus added to all test tubes in both runs. All tubes were run in triplicate alongside a control tube without virus, consisting of just Hela cells. Due to unwanted background pickup caused by the Taqman machine noticed in previous experiments with the polystyrene beads heparin binding, serial dilutions were performed on the viral samples to determine if any significant binding occurred.

In the first experiment the viral samples were diluted 1:1, 1:10, and 1:100. All viral samples that were diluted 1:100 were DRP titer around the same concentration as the cell control tube. This suggests that the samples must contain greater than  $10^7$  amount of virus in order to give accurate results without interference with the CMV probes or background clutter. Wild type AAV2, AAV2 R585A R588A VP1,3 VP2, and a control were incubated in the harvested Hela cells at  $4^\circ\text{C}$  for 60 minutes allowing for binding to occur. The samples were washed three times to remove any unbound virus

and the cells were lysed with a lysis buffer. All of the clarified lysates were Taqman/DRP analyzed for concentrations. Figure 9 demonstrates a linear chart of the serial dilutions increasing in concentration from left to right. As the amount of virus increased, the amount of virus recovered for wild type AAV2 also increased. The heparan mutants continued to stay close to the cell control concentrations. This concludes that the heparan mutants did not successfully bind to the Hela cells and appeared similar to background being picked up by Taqman. The cell control should have come back with an almost zero concentration due to the fact that no virus was present and thus no CMV promoters were present to be picked up by the CMV probe.

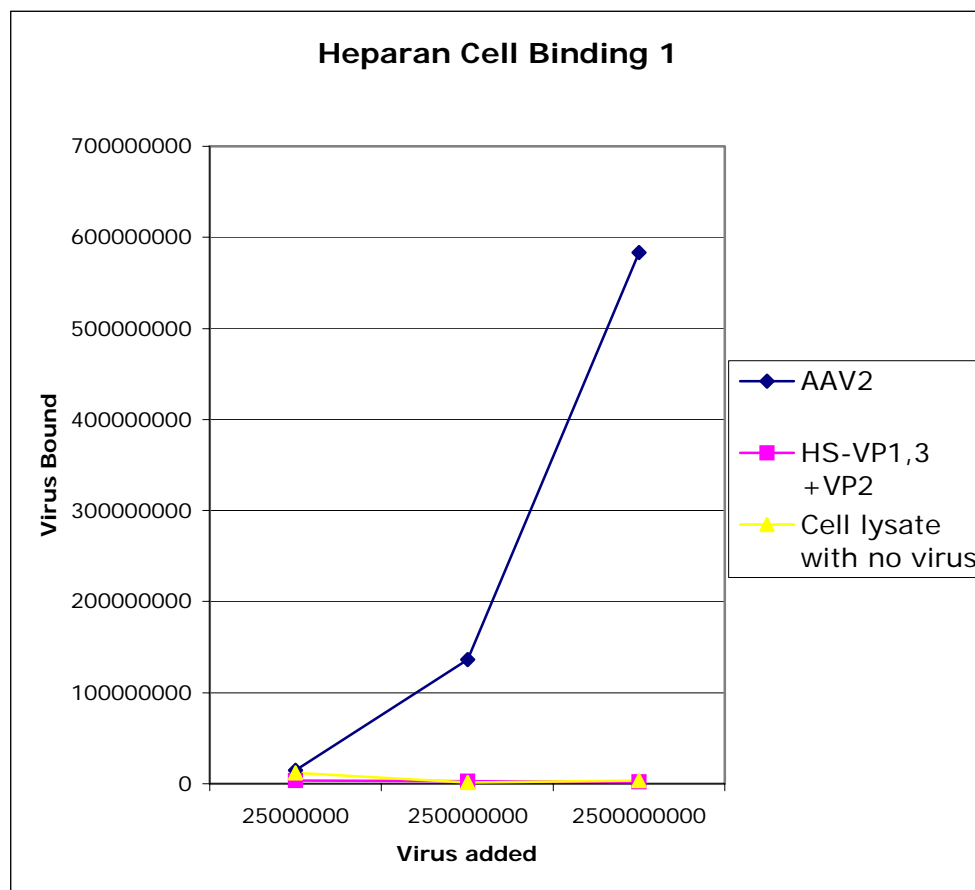


Figure 9: Heparan Cell Binding experiment 1. Serial dilutions of the viral preps were from left to right, 1:100, 1:10, and 1:1. As the concentration increased of the viral preps, the amount of cellular binding that did occur also increased. Wild type AAV2 was the only virus that successfully bound to the Hela cells and the heparan mutants and cell control can back similar to background pickup.



In the second experiment the viral samples were diluted 1:1, 1:10 and 1:50.

Since all viral samples from the first experiment that was diluted 1:100 came back as just background pickup, a 1:50 dilution was undertaken to see if improvements could be made. The amount of Hela cells was also increased from previous concentrations of  $2.5 \times 10^5$  cells to  $5.0 \times 10^5$  cells so a larger pellet could be seen to control the amount of cells that was not aspirated off by accident. The viral samples were treated the same as the first experiment and results are found in Figure 10 below.

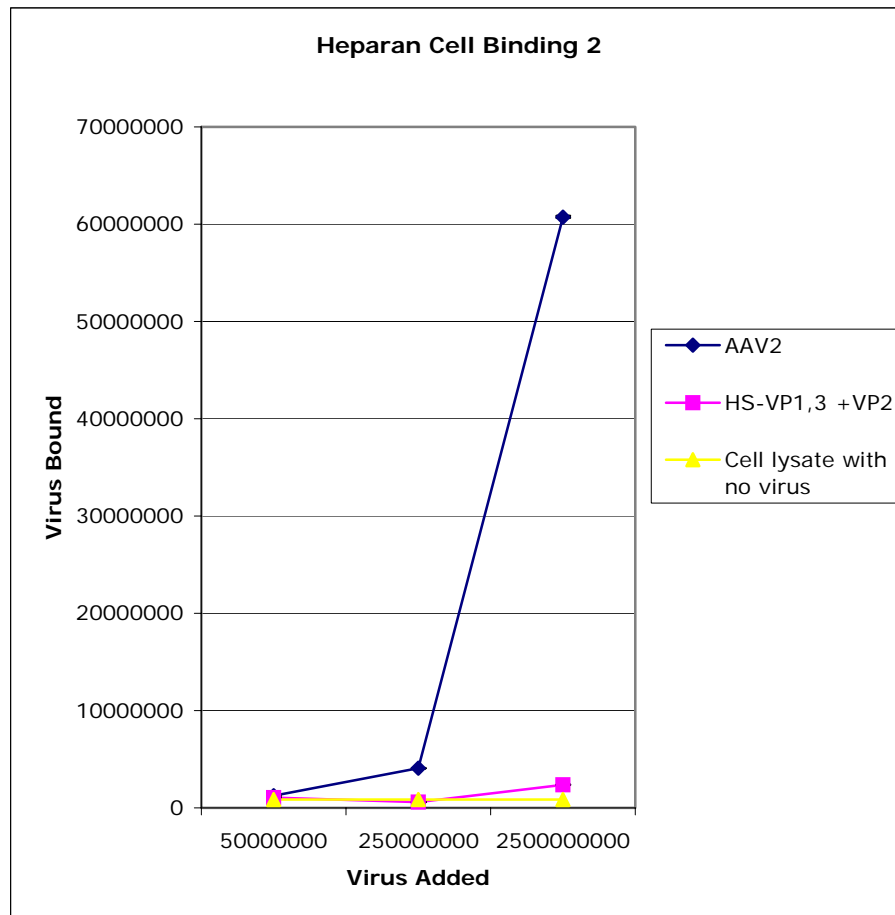


Figure 10: Heparan Cell Binding experiment 2. Serial dilutions were performed on the viral samples from left to right as 1:50, 1:10, and 1:1. As concluded from the first experiment as the amount of virus concentration increases, the amount of background pickup decreases and viruses bound increases. AAV2 again was the only significant Hela cell binding when compared to the heparan mutants.

The ability of mutants to bind heparan sulfate on HeLa cells was assessed in an affinity assay in combination with a competitor. This affinity assay will determine the amount of specific binding that is occurring to the HeLa cells as compared to non-specific binding. Specific binding of wild-type AAV should occur in the absence of a competitor. AAV binds to its natural receptor HSPG that is expressed on the surface of HeLa cells. AAV should not demonstrate specific binding in the presence of a competitor in a 100-fold excess that is competing for the same HSPG binding. The R585A, R588A AAV should demonstrate non-specific binding since its ability to bind to its natural receptor, HSPG, has been eliminated.

The protocol was set up similar to the cell binding assay, only in conjunction with the AAV vectors made in Dr. Bartlett's laboratory with a CMV promoter, another AAV vector without the CMV promoter was added. The competitor virus called CC119 was a gift from Sferra's lab, which was a rAAV2 Eflα eGFP at a concentration of  $7.6 \times 10^{12}$  DRP/ml. To analyze the concentration of our viral vectors through the Taqman/DRP titer, a CMV probe is utilized. The CMV probe picked up any virus that bound to the HeLa cells and a concentration would be given. This value is further calculated out to determine the active concentration of the virus of every particle (DRP) per microliters.

The viruses were tested to determine if they could bind to the surface of the HeLa cells in the presence of a competitor in 100-fold excess. The samples were allowed to bind to the HeLa cells at 4°C for 120 minutes. After cell lysis, the lysates was analyzed by a Taqman/DRP titer to determine the amount of virus in the solution. AAV2 did not bind as well to the HeLa cells similar to the heparan mutants in the cellular binding assay

when the competitor virus was added. Figure 11 demonstrates how AAV2 bound approximately 32% of the time to the Hela cells in the absence of a competitor. When competitors to the same heparan binding receptors were added in excess with AAV2, AAV2 only bound approximately 2% of the time. The heparan mutants really did not bind either of the times regardless of the competitor.

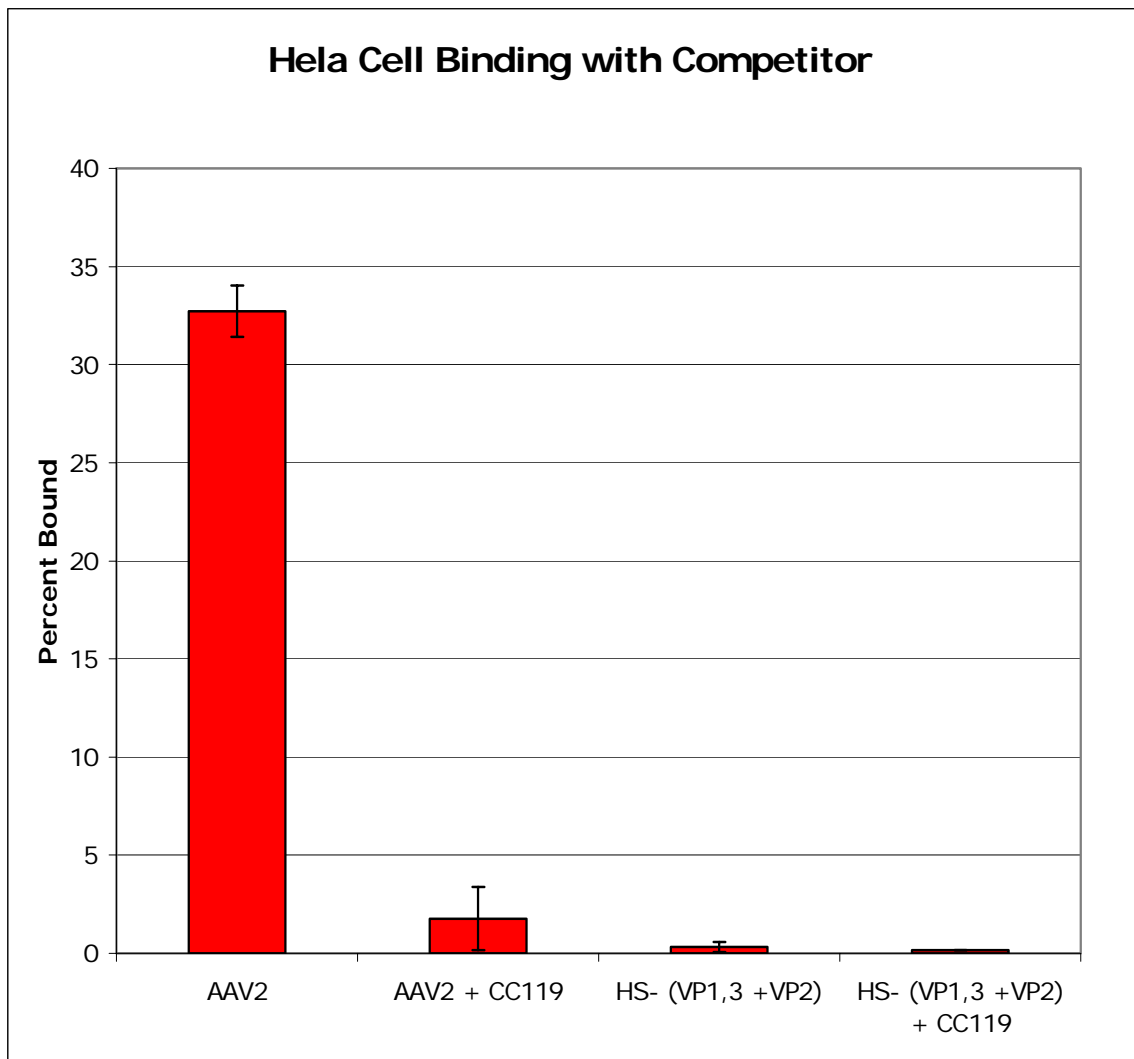


Figure 11: Hela Cell Binding with competitor. The CC119 competitor was added alongside the viral preps to compete for binding receptors. CC119 is a rAAV2 Efl $\alpha$  eGFP that was added in 100-fold excess of all of the viral preps. Alone, AAV2 bound 32% of the time and AAV2 R585A R588A VP1,3 plus VP2 bound 0.32%. In the presence of a competitor neither virus bound as well.

These preliminary findings suggest that AAV2 does bind to heparan, and that the rAAV2 mutants were deficient in receptor binding. AAV2 demonstrates specific binding in the absence of a competitor for the same receptor HSPG. In the presence of a competitor, AAV2 demonstrates non-specific binding to the HeLa cells. The heparan mutants demonstrated non-specific binding to the HeLa cells in both incidences when a competitor was present and without. This truly demonstrates the elimination of HSPG binding of the AAV vector. Eliminating the heparan binding portion of the AAV2 vector can lead to successful elimination of unwanted gene transfer and expression and the retargeting of the rAAV2 vector to tissues that were once thought untargetable.

## CHAPTER 5

### DISCUSSION AND CONCLUSION

Modifying plasmids with R585A R588A point mutations has successfully eliminated heparan sulfate proteoglycan binding of the AAV2 vector. Eliminating the binding to natural receptor of AAV2 and introduction of new peptides improves the gene transfer and expression of the virus to tissues of interest. AAV2 has the ability to be the solution to all of the problems of gene therapy. Its ability to invade both dividing and non-dividing cells, long term gene expression, reproducibility of high titers, and lack of pathogenicity makes AAV2 a suitable vector for gene therapy. Retargeting AAV2 to tissues of interests can improve the effectiveness and efficiency of gene therapy for treating cancers and genetic disorders.

In vitro studies are conclusive that AAV2 with mutations at the R585A R588A positions does not bind heparin on polystyrene beads, heparan expressing cells, and specific binding has been eliminated in the presence of a competitor. The R585A R588A AAV2 mutation does not infect cells, which is thought to be or probably due to the lack of binding to the heparan expressing Hela cells. What is not apparent is whether the modified viral vectors can bind to cells expressing their new peptide receptors. Future studies will be tested on tissue culture cells that have been known to express the various receptor sites on their surface. VEGF receptor binding ligands were added to the

modified AAV2 vectors deficient in receptor binding. Once tissue tropisms of the AAV vector are deficient in receptor binding and retargeted to new wanted receptors, in vivo studies can be investigated.

Future studies will examine the in vivo dissemination of the viral vectors deficient in natural receptor binding developed in our laboratory. The modified vectors should transduce cells with matching viral receptors. Gene expression of the modified heparan mutants will have to be monitored and the levels of the vectors that are expressed determined. The objectives of the original proposal were to test the modified heparan mutants mainly in vivo. Goals were set high, but were not unobtainable. As with any research topic, unforeseen obstacles surfaced. Troubleshooting and pondering the problems that went wrong helped me to understand the reasoning behind what all I did. If everything went as planned, I could have completed a lot more objectives originally proposed, but then it would not be research. Albert Einstein already foresaw the pitfalls in my research. He once said, "If we knew what it was we were doing, than it would not be called research, would it?" If research was easy to complete in a short amount of time, than everyone would be able to fix all of the problems and cancers in the world. I was given a wonderful opportunity to complete something once believed unimaginable. With all of my new gained knowledge I acquired from my research, I feel even more lost in understanding it. As the old saying goes, "The more you know, the more you realize how much you don't know."

## CHAPTER 6

### BIBLIOGRAPHY

1. Bartlett, J.S., Wilcher, R., Samulski, R. "Infectious Entry Pathway of Adeno-Associated Virus and Adeno-Associated Virus Vectors." *Journal of Virology*. **74**(6): 2777-2785 (2000).
2. Shi, W., Bartlett, J.S. "RGD Inclusion in VP3 Provides Adeno-Associated Virus Type 2 (AAV2)- Based Vectors with a Heparan Sulfate-Independent Cell Entry Mechanism". *American Society for Gene Therapy: Molecular Therapy*. **7-4**: 515-525 (2003).
3. Opie, S. R., K. W. Warrington, Jr., et al. "Identification of amino acid residues in the capsid proteins of adeno-associated virus type 2 that contribute to heparan sulfate proteoglycan binding." *Journal of Virology*. **77**(12): 6995-7006 (2003).
4. Buller, R.M., Rose, J.A.. "Characterization of adenovirus-associated virus – induced polypeptides in KB cells." *Journal of Virology*. **25**:331-338 (1978).
5. Shi, W., et al. "Insertional mutagenesis of the adeno-associated virus type 2 (AAV2) capsid gene and generation of AAV2 vectors targeted to alternative cell- surface receptors." *Human Gene Therapy* **12**:1697-1711(2001).
6. Warrington, K., et al. " Adeno-associated Virus Type 2 VP2 Capsid Protein Is Nonessential and Can Tolerate Large Peptide Insertions at its N Terminus." *Journal of Virology*. **78**(12): 6595-6609 (2004).
7. Xie, Q., W. Bu, et al. "The atomic structure of adeno-associated virus (AAV2), a vector for human gene therapy." *Proc Natl Acad Sci USA* **99**(16): 10405-10 (2002).
8. Kronenberg, S, et al. "Electron cryo-microscopy and image reconstruction of adeno associated virus type 2 empty capsids." *EMBO reports* **2**(11): 997–1002 (2001).
9. Büchen-Osmond, C. "*Dependovirus*." 2002. ICTVdB The Universal Virus Database. 18 March 2005.  
<<http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdb/index.htm>>.
10. Rabinowitz, J., Samulski, R.J. "Adeno-associated virus expression systems for gene transfer". *Current Opinion in Biotechnology*. **9**:470-475 (1998).
11. Feuder, E., Alwis, M., Thrasher, A., Ali, R., Fauser, S. "Optimization of recombinant adeno-associated virus production using an herpes simplex

virus amplicon system.” *Journal of Virological Methods*. **96**, 97-105 (2001).

12. Girod, A., M. Ried, et al. “Genetic capsid modifications allow efficient re-targeting of adeno-associated virus type 2[published erratum appears in Nat Med 1999 Dec; 5(12): 1438].” *Nature Medicine* **5**(9): 1052-6 (1999).
13. Müller, O., et al. “Random peptide libraries displayed on adeno-associated virus to select for targeted gene therapy vectors.” *Nature Biotechnology*. **21**(9): 1040-1046 (2003).
14. Kern, A., K., Schmidt, et al. “Identification of a heparin-binding motif on adeno-associated virus type 2 capsids.” *Journal of Virology*. **77**(20): 11072-11081 (2003).
15. Nicklin, S.A., et al. “Efficient and selective AAV2-mediated gene transfer directed to human vascular endothelial cells.” *Mol. Ther.* **4**(3) : 174-181(2001).
16. Xiao, X., Li, J., and Samulski, R. “Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus.” *Journal of Virology*. **72**: 2224-2232 (1998).
17. Li, J., Samulski, R.j., and Xiao, X. “Role for highly regulated rep gene expression in adeno-associated virus vector production.” *Journal of Virology*. **71**: 5236-5243 (1997).
18. Clark, K., Voulgaropoulou, F., and Johnson, P. “A stable cell line carrying adenovirus-inducible rep and cap genes allows for infectivity titration of adeno-associated virus vectors.” *Gene Therapy*. **3**:1124-1132. (1996).
19. Jordan, M., et al. “Transfecting mammalian cells: optimization of critical parameters affecting calcium-phosphate precipitate formation.” *Nucleic Acids Res.* **24**: 596-601(1996).
20. Zolotukhin, S. “Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield.” *Gene Therapy* **6**: 973-985. (1999).
21. Clark, K. R., Liu, X., McGrath, J.P., Johnson, P.R., “Highly purified recombinant adeno-associated virus vectors are biologically active and free of detectable helper and wild-type viruses. *Human Gene Therapy* **10**, 1031-1039.